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Homeobox Genes Exhibit Evolutionary Conserved Regionalization in the Central Nervous System of an Ascidian Larva

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ABSTRACT—Animals in each subgroup of the phylum Chordata exhibit a similar process by which they form a tubular central nervous system (CNS). However, little is known about spatial relationship among the CNSs of chordates; vertebrates, cephalochordates and urochordates (tunicates). Ascidians constitute a major animal group in the subphylum Urochordata. In the present study, we examined the expression patterns of *labial* and *orthodenticle* related genes of the ascidian, *Halocynthia roretzi*, in the developing larval CNS. These homeobox genes exhibited region-specific expression patterns that are strikingly similar to those of murine *Hoxb-1* and *Otx2*. The regionalization as characterized by the expression of these genes supports the division of the ascidian larval CNS suggested by the previous morphological studies. Furthermore, conservation of the expression pattern of the homeobox genes suggests that such regionalization occurred in the CNS of a putative common ancestor of chordates.

INTRODUCTION

Ascidian embryos form a tubular CNS in a similar way to that of vertebrates. The ascidian larval CNS can be divided into two parts, a prosencephalon over a gut primordium and a deuterencephalon underlain by a notochord (Katz, 1983). Nicol and Meinertzhagen (1991) distinguished four regions in the larval CNS of *Ciona* based on the composition of cell types, which are the sensory vesicle and neck in the prosencephalon and the visceral ganglion and spinal cord in the deuterencephalon. The sensory vesicle is further subdivided into three regions. These descriptions show that the ascidian CNS has its own characteristic morphology and therefore the spatial relationship in the CNS between vertebrates and ascidians is difficult to be understood by morphology.

A number of homeobox genes, including HOM-C genes of *Drosophila* as excellent examples, have been found to play key roles in regional specification in developmental fields of metazoan embryos. In the developing murine CNS, Hox genes define segmental identities of the rhombomeres that are developmental subdivisions of the hindbrain (McGinnis and Krumlauf, 1992). In the more anterior regions, fore- and mid-brain, homeobox genes of other class such as *Emx* and *Otx* show spatially restricted expression along the anteroposterior (A-P) axis (Holland *et al.*, 1992; Simeone *et al.*, 1992). In the present study, we compare the expression pattern of *Hroth*, an ascidian homeobox gene related to *Otx*, with that of *HrHox-1* in the developing larval CNS. In the light of the present findings, we discuss regionalization of the ascidian larval CNS and spatial relationship between the CNSs of ascidians and vertebrates.

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MATERIALS AND METHODS

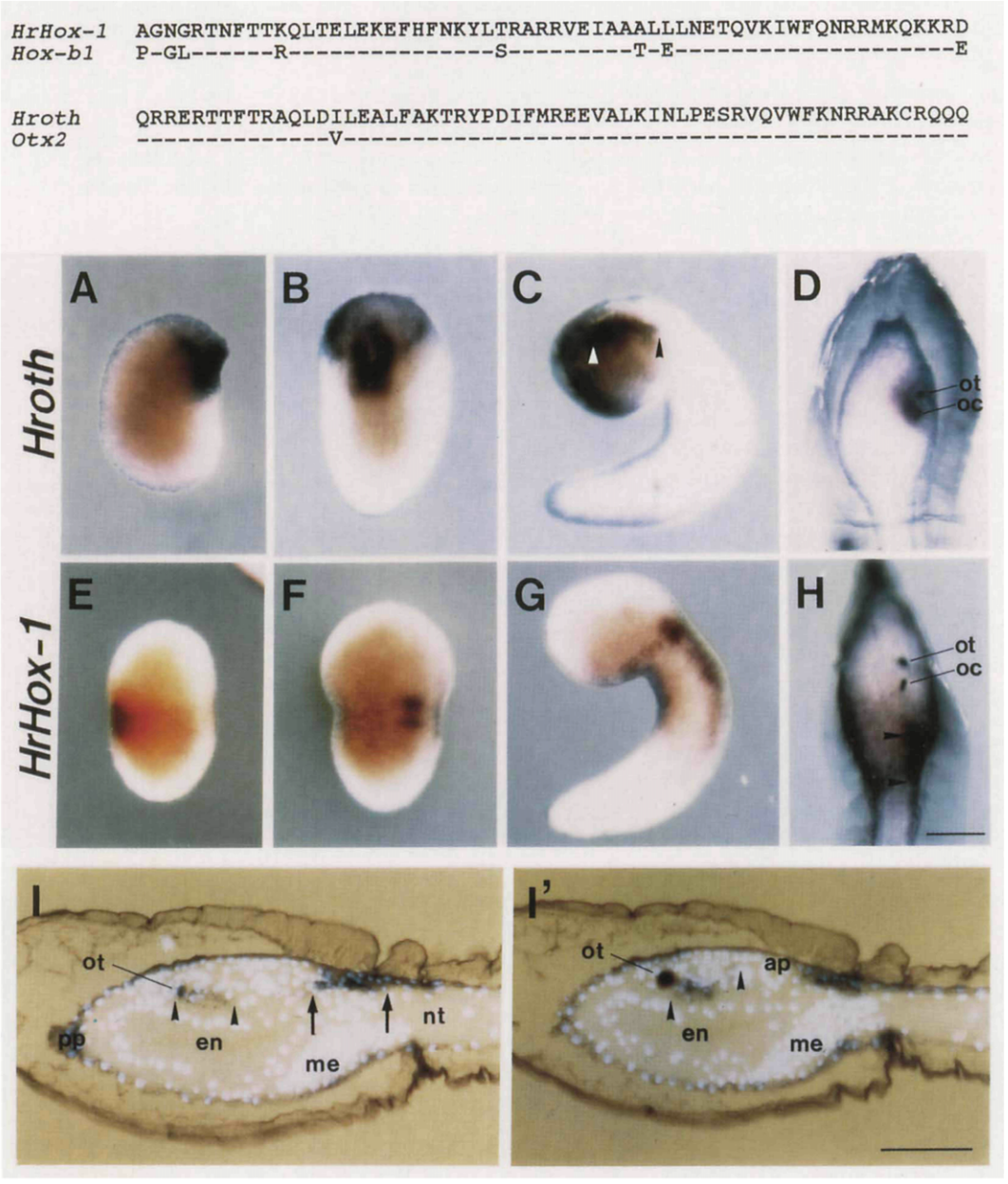
Embryos of the ascidian, *Halocynthia roretzi*, were raised as described previously (Katsuyama *et al.*, 1995). Whole mount *in situ* hybridization was carried out according to the procedure described by Wada *et al.* (1995). Probes used in this study were the *HrHox-1* cDNA clone, HH-1f (Katsuyama *et al.*, 1995) and a 2.1 kb cDNA clone of *Hroth* (Wada *et al.*, in preparation). Detailed procedures for isolation of *Hroth* gene will be described elsewhere (Wada *et al.*, in preparation). Briefly, a gene fragment of *Hroth* was obtained by PCR using *Halocynthia* gastrula cDNA and primers for the *bicoid* class homeobox and used as a probe to isolate the clone from a 64-cell stage cDNA library.

RESULTS AND DISCUSSION

Previously we found that *HrHox-1*, the ascidian Hox gene structurally related to *labial* of *Drosophila* and Hox genes of paralogous subgroup 1 of vertebrates, shows the expression pattern similar to that of the murine *Hoxb-1* gene in the developing ascidian CNS (Fig. 1) (Katsuyama *et al.*, 1995). Then we isolated and examined the expression pattern of an ascidian homologue of *orthodenticle*, designated *Hroth* (*Halocynthia roretzi orthodenticle* homologue) (Fig. 1). Here our attention will be focused on expression of *Hroth* in the CNS. At the neurula and tailbud stages, *Hroth* is expressed in

the anterior region of the neural fold, closing to form a neural tube (Fig. 1A). This expression is detected as two short stripes running parallel along the A-P axis (Fig. 1B). At the tailbud stage, these become a single stripe (Fig. 1C). At the larva stage, expression of *Hroth* is observed in the sensory vesicle, surrounding two types of pigment cells, the otolith and the ocellus (Fig. 1D, 1I, 1I').

HrHox-1 expression in the CNS becomes detectable from the tailbud stage onward in the region posterior to the expression domain of *Hroth* (Fig. 1F). Thus, the two expression domains align along the A-P axis in the developing CNS from this stage (Fig. 1B, 1F). The anterior border of *HrHox-1* expression remains fixed at the level rostral to the anterior tip of the notochord. Thus, the two expression domains do not overlap, keeping a space between them throughout the embryogenesis. The expression patterns of both genes in the CNS are schematically illustrated and compared with those of murine counterparts in Fig. 2. This comparison shows striking similarity between expression patterns of the two sets of related genes in the CNS of the ascidian and mouse. Expressions of the murine *Otx* genes are restricted to presumptive fore- and mid-brain region in the CNS (Simeone *et al.*, 1992), while *Hroth* is expressed in the anterior part of the ascidian CNS. Similarly, expression of *Hoxb-1* is restricted to the rhombomere 4 of 9.5 d.p.c. mouse embryo (Frohman *et al.*, 1990; Murphy and Hill, 1991), while *HrHox-1* expression is restricted to the region encompassing the level of the atrial primordium to the junction of the trunk and tail in the ascidian



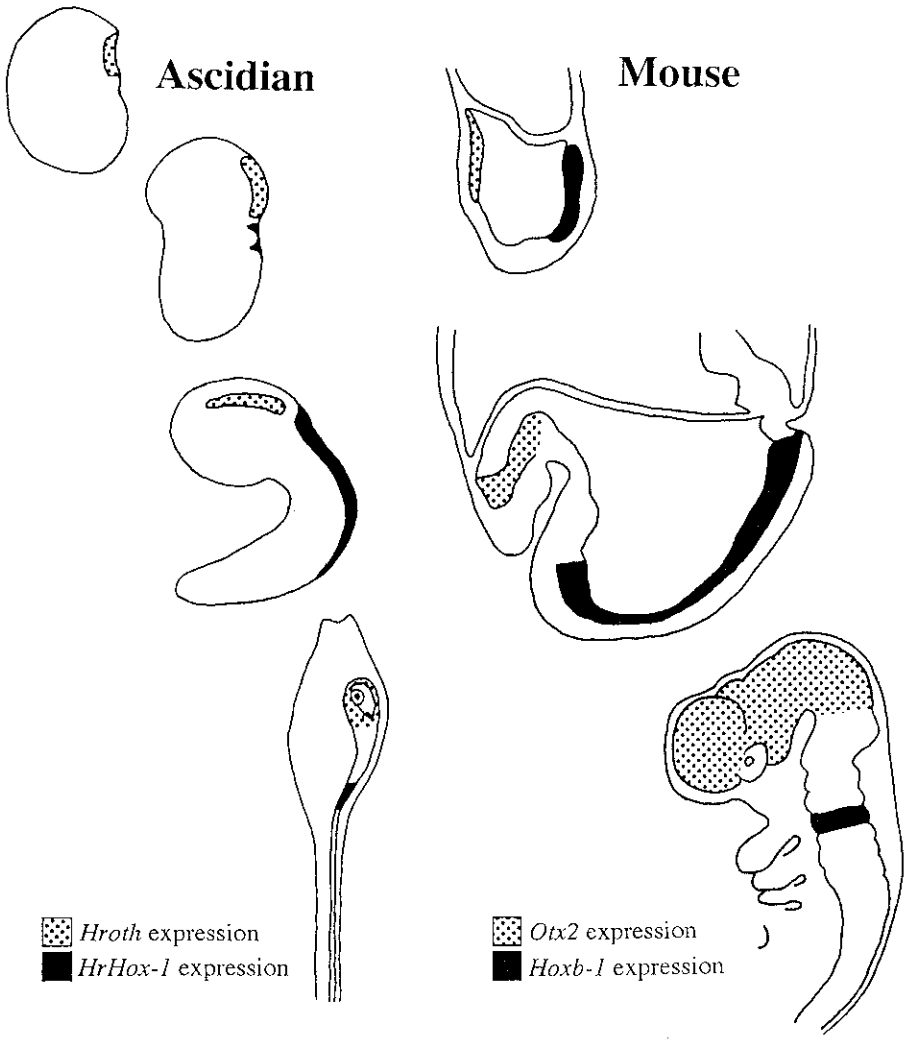


Fig. 2. Summary of the expression patterns of *Hroth* and *HrHox-1* and comparison with those of putative murine homologues, *Otx2* and *Hoxb-1*. Expression patterns of two murine homeobox genes are depicted according to Simeone *et al.* (1992), Frohman *et al.* (1990) and Murphy and Hill (1991). In this illustration, only the expression in the CNS is shown as regards the ascidian homeobox genes. Ascidian embryos in the left side are, from top to bottom, an early neurula, an early tailbud, a late tailbud and a swimming larva. In the right side, mouse embryos of, from top to bottom, 7.5, 8.5 and 9.5 d.p.c. are drawn.

Fig. 1. Amino acid sequences of the homeodomains and expression patterns of *HrHox-1* and *Hroth*. Upper shows amino acid sequences of the homeodomains of *HrHox-1* and *Hroth* aligned with putative murine homologues, *Hoxb-1* and *Otx2*. Bars indicate identical amino acid residues. Lower pictures show expression of *Hroth* (A-D) and *HrHox-1* (E-H) as revealed by whole mount *in situ* hybridization and sections of a specimen of the larva after whole mount *in situ* hybridization (I and I'). (A) A lateral view of an early neurula. Dorsal is to the right. Expression of *Hroth* is observed at the neural fold. (B) A dorsal view of an early tailbud stage embryo. *Hroth* expression is detected as two stripes in the anterior portion of the neural fold closing to form a neural tube. Expression of *Hroth* is also observed in the anterior epidermis of the embryo. (C) A lateral view of a late tailbud stage embryo. Because the midline of the embryo slightly twists to the left at this stage, the dorsal-most region is observed in this picture. Expression of *Hroth* is observed as a stripe (indicated by arrowheads) in the anterior region of the neural tube. Expression is also observed in the epidermis in the anterior portion of the trunk. (D) A lateral view of a swimming larva. Expression of *Hroth* is observed around the sensory vesicle. Two types of pigment cells in the sensory vesicle are indicated by ot, otolith and oc, ocellus. Expression of *Hroth* is also detected at the papillae. Tunic surrounding the larval body is nonspecifically stained. (E) An early neurula. Expression of *HrHox-1* is not observed in the neural tube forming region at this stage, while expression is detected in the epidermis around middle portion of the embryo. (F) An early tailbud stage embryo. Expression of *HrHox-1* in the neural tube is clearly observed from this stage on, which is posterior to the junction of tail and trunk. Expression of *HrHox-1* in epidermis is also detected. (G) A late tailbud stage embryo. Anterior border of *HrHox-1* expression in the neural tube resides at the junction of the trunk and tail. Expression of *HrHox-1* extends toward posterior forming a gradient along the spinal cord. Expression in the epidermis remains. (H) A dorsolateral view of a swimming larva. Expression of *HrHox-1* in the CNS is observed along the midline in the posterior region of the trunk as indicated by arrowheads. Expression is not observed in the spinal cord. Epidermal expression of *HrHox-1* is observed over a posterior portion of the larval trunk. (I and I') Expression of *HrHox-1* and *Hroth* in a swimming larva. After whole mount *in situ* hybridization using *HrHox-1* and *Hroth* probes simultaneously, a specimen was saggitally sectioned and counterstained with DAPI. *HrHox-1* is expressed in the seven cells in a line (between the arrows in picture I) which are included in the visceral ganglion. *Hroth* is expressed in the anterior two thirds of the sensory vesicle (between the arrowheads in picture I or I'). ap, atrial primordium; en, endoderm; me, mesenchyme; nt, notochord; ot, otolith; pp, papillae. Magnification is the same through A-H. Scale bars indicate 100 μm.

larval CNS (Fig. 1H, 1I, 1I').

Expression of these homeobox genes characterizes four regions in the ascidian larval CNS; from rostral to caudal, the *Hroth* expressing region, the intervening region, the *HrHox-1* expressing region and the spinal cord, the region posterior to these. *Hroth* and *HrHox-1* expressing regions are included in the prosencephalon and the deuterencephalon of the ascidian larval CNS, respectively, as designated by Katz (1983). In vertebrates, a prosencephalon and a deuterencephalon are the regions where forebrain and hindbrain develop, respectively. In this regard, the expression pattern of *Hroth* and *HrHox-1* in the CNS supports the designation by Katz (1983). Considering the histological division by Nicol and Meinertzhagen (1991), the *HrHox-1* and *Hroth* expressing domains correspond to the visceral ganglion and the sensory vesicle except the posterior sensory vesicle that is the posterior most subdivision of the sensory vesicle. The intervening region which includes the posterior sensory vesicle and the neck, might be characterized by the expression of other genes such as an *engrailed* cognate.

Recent molecular analysis and morphological comparison have shown the phylogenetic relationship among subphyla of chordates (Brusca and Brusca, 1990; Wada and Satoh, 1994), which implies that chordates share a common ancestor and that ascidians diverged first from the lineage to vertebrates. Thus, similarity between the expression patterns of *HrHox-1* and *Hroth* and those of their vertebrate counterparts suggests that such molecular regionalization in the CNS along the A-P axis occurred in a common ancestor of the ascidians and vertebrates, that is a putative earliest chordate.

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